(2)

THE PROCURING AND PROCESSING

OF HUMAN CADAVERIC BONE MARROW

NMRI 90-62

UNITED STATES NAVY TISSUE BANK
IMMUNOBIOLOGY AND TRANSPLANTATION DEPARTMENT
NAVAL MEDICAL RESEARCH INSTITUTE
NATIONAL NAVAL MEDICAL CENTER
BETHESDA, MARYLAND
20814-5055

Written and Compiled By: Timothy R. Faloon, HM1/USN

Human cadaveric vertebral bodies offer a unique source of bone marrow for use in research and clinical transplantation protocols. Techniques to enhance maximum cell recovery from this tissue while maintaining a high viability of marrow matrix are constantly under revision. This manual will focus on the techniques utilized primarily at this facility. It will address the procuring, processing, freezing and thawing of bone marrow which we have found to produce maximum yields.

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FOREWORD

The goal of the U.S. Navy Tissue Bank seeks to preserve life. Its research is directed toward numerous afflictions which result in the loss of function, and in all too many cases, loss of life.

In the event of the death of a patient, considerations should be given to what benefit, if any, can be accrued to medical knowledge and thus to future patients. Irrespective of the cause of death, these thoughts should come to mind.

If irreversible global damage to the brain has occurred, while respiration and circulation have been maintained, tissue donation should be considered. Tissue will not be collected from donors who have had a history of malignancy, serious infection, or a disease of unknown etiology. In the event that tissue donation cannot be accomplished, officials attached to State government or local medical schools can be of assistance in arranging for "whole body" donation for anatomical study.

To be of clinical value, bone marrow must be obtained within specified time limits: upon cessation of the heartbeat and after two flat electroencephalograms (EEG's) run 13 hours apart; bone marrow, for research and transplantation, must be obtained within 12 hours after death. Prior to removal of any tissues, **Authorization for Autopsy**, SF 523, and **Authorization for Tissue Donation**, SF 523-B, must be signed by the next-of-kin and two adults as witnesses, Appendix (a) and (b). It should be equally emphasized that before the removed tissue may be used clinically, the cause of death must be determined through an unrestricted diagnostic post-mortem. In view of the inherently complex administrative problems associated with maintaining proper relationships with the bereaved next-of-kin, and complying with pertinent laws and regulations, it is essential and intended that all involved personnel cooperate to the fullest extent to ensure that an acceptable, successful, and efficient program of tissue donation be attained.

The Navy Tissue Bank will respond on a 24-hour basis to notification of the existence of a possible donor. Consultation about the advisability and procedural considerations of tissue donation is available. The Donor Criteria is a list of contraindications to tissue donation. Ideal donors are those who die acutely from trauma, cardiovascular, or central nervous system causes; when donation can be accomplished according to time limits placed on the various types of tissues requested; and when tissue procurement does not interfere with memorial services and viewings. As members of society become more knowledgeable about transplantation, obtaining permission for tissue and organ donation will become routine.

DONOR CRITERIA

Bone marrow shall not be collected or requested from donors with any of the following conditions:

Infections

1. Septicemia (from any cause) at time of death
2. Systemic mycosis, or history of systemic mycosis
3. Meningitis or encephalitis
4. Leprosy
5. Active viral disease, or past history of chronic viral disease
6. Active tuberculosis, or history of tuberculosis
7. Active hepatitis, or history of hepatitis
8. Active malaria, or history of malaria
9. Active syphilis, or history of syphilis
10. AIDS, or history of infection with HIV/HTLV-III/LAV (e.g., antibody positive)

Malignancies - except primary basal cell tumor of the skin considered cured following excision (decision of the Tissue Bank Watch Officer) anywhere in the body; or active disease or history of malignancy.

1. Lymphoma
2. Leukemia
3. Sarcoma
4. Carcinoma

Autoimmune diseases and conditions of unknown etiology

1. Rheumatoid arthritis
2. Systemic lupus erythematosis
3. Polyarthritis nodosa
4. Sarcoidosis
5. Myasthenia gravis

Miscellaneous

1. Patients who have had a tracheostomy or have been on a respirator more than 96 hours.
2. Chronic parenteral drug use affecting tissues to be procured (e.g., steroids).
3. Irradiation to tissues to be procured.
4. Toxic substance exposure sufficient to affect tissues to be procured.

Bone marrow collection may be accomplished up to twelve (12) hours after death, provided the donor's body has been adequately refrigerated for this period of time.

ASEPTIC PRECAUTIONS

The operating room of the Tissue Bank is reserved exclusively for the procurement and processing of human tissue. The design of the operating room includes a HEPA air filtration system, stainless steel furniture, stainless steel walls, a short-wave ultraviolet light system which minimizes the presence of surface ambient flora, and a single piece tile floor void of seams and creases. In the case of cadaveric donors, you can neither assume an intact immune system nor expect that colonization of a wound at the time of the tissue procurement will be countered by an appropriate immune response. Therefore, the technicians who perform the tissue excision must be skilled in the rapid removal of tissue in order to reduce contamination. Close scrutiny is maintained in order to detect any break in technique during the tissue excision. The appropriate surgical attire (e.g., surgical scrubs, hats, masks, and shoe covers) will be worn by all personnel in the operating room.

Method

The donor will first be prepared in the adjacent preparation area (primary skin prep and hair removal) and then transferred to the operating table, which has been brought out of the operating room. The gurney will <u>not</u> be brought into the operating room because of the concern for contamination being transferred from the wheels of the gurney to the operating room spaces. A sheet is soaked with the appropriate disinfectant and placed in front of the entrance to the operating room to prevent the tracking of particulate and bacterial contamination into the operating room.

There will be occasions when tissue procurements will be performed in the operating rooms of other facilities. In these situations, a fully equipped procurement team, consisting of four to five members, will be dispatched to the facility by the most expedient means of transportation available. All functions will be with the expressed consent of the hosting facility. In general, after obtaining permission for the tissue procurement, the remains will be transported to the operating room and preparation of the donor and the tissue procurement will proceed in a routine fashion. It is the responsibility of the procurement team to re-establish the cleanliness and order of the operating room used in the hosting facility.

DONOR QUALITY CONTROL

Blood Specimens

Blood for serological and bacteriological tests is obtained in the workroom prior to the procurement procedure. If vertebral bodies are sent by an affiliate hospital, these blood studies are required prior to commencing processing procedures.

The blood is obtained via the jugular vein, subclavian vein, an intracardiac puncture. or femoral vein. The chest, neck, or groin is first prepped with Betadine soap and painted with Betadine scrub. Using a fifty (50) milliliter syringe and an 18 gauge needle, 50 mls. of blood is drawn from the chosen vessel.

Blood Cultures

Approximately five (5) mls. of blood is injected into each of four (4) blood culture bottles (2 aerobic and 2 anaerobic), ensuring sterility and changing needles with each bottle.

VDRL and HAA

Approximately seven (7) mls. of blood is injected into each of two red-topped tubes (a separate tube is used for each test). Each tube is labeled with the donor's name, social security number or identification number, date, and test requested. These tubes, along with appropriate serology chits are taken to the STAT lab as soon as possible.

HLA

Approximately seven (7) mls. of blood is then injected into a green-topped tube, labeled with patient information, and placed in the Tissue Bank Tissue Typing Laboratory for HLA typing.

HIV/HTLV-III/LAV

Approximately ten (10) mls. of blood is injected into a red-topped tube. The tube is also labeled with donor's name, social security or identification number, date, and test required. The remaining blood is injected into another red-topped tube, centrifuged, serum decanted, labeled with patient information, and placed in the Tissue Bank blood refrigerator. This specimen is to be resubmitted to the main laboratory in the event that the original specimens for serology are lost, misplaced, or a repeat of the original test for any of the above is required.

Diagnostic Post-Mortem

To ensure that the donor criteria has been complied with, a diagnostic post-mortem must be completed and reviewed prior to the utilization of all tissues previously procured.

BACTERIAL QUALITY CONTROLS

Frequent and various checks are made during the procurement and processing of tissue to ascertain that all methods of reducing contamination are reasonably effective.

Sterilizer Quality Control

The steam autoclaves used for the sterilization of supplies are checked with each use for their effectiveness in killing all organisms. Steam indicator strips are placed in all packs, along with a tube containing Bacillus stearothermophilus spores. At the completion of the sterilization cycle the indicator strips are checked for the penetration of the steam and the tube containing the spores are incubated for recovery of any surviving organisms.

Operating Room Cultures

To determine the gross quantity of organisms in the circulating air, broth-wetted culture plates are exposed to room air for approximately twenty (20) minutes. Slit air sampling is done to determine air-borne contamination. Swab cultures are also done on all flat surfaces to determine the residual bacteria in the operating room. This culturing is performed twice a month.

Handling of Tissue

All handling of tissue should be performed aseptically. The handling of tissue with gloved hands should be minimized in favor of the use of sterile instruments. Packaging and wrapping should also be performed with sterile containers and sterile linen.

PREPARATION OF THE OPERATING ROOM FOR AN ASEPTIC POST-MORTEM TISSUE EXCISION

Cleaning the Operating Room

All furniture, walls, floor space, ceilings, and overhead lights will be washed down with a detergent disinfectant solution.

Stocking the Operating Room

To ensure the cleanliness and expedient procurement of tissue, all equipment associated with the procedure will be kept in enclosed cabinets within the operating room.

Stock solutions to be kept on hand in the operating room include the following:

1. Sterile 0.9% NaCl for irrigation
2. Sterile H2O
3. One gallon of Betadine Solution
4. One gallon of Betadine Scrub
5. One gallon of 70% Isopropyl alcohol
6. One gallon of BacDown scrub solution
7. Sterile Bone Marrow reagents/solutions
8. Aerobic/Anaerobic culture bottles

The Preparation of Sterile Tables and Trays

Since the entire case is treated as two separate procedures (the procurement of the bone marrow as one and the processing of the bone marrow as the other), preparation must be made so that the linen, instruments, and various supplies are available and that each procedure be treated as a separate operation in order to ensure no cross-contamination.

1. Sterile linen supplies

Sterile linen supplies and gloves are arranged on the back table. This table contains drapes, towels, sterile gowns, and gloves for the entire procedure. Each procedure will have a separate back table set-up.

2. Instruments and sterile supplies

Instruments and sterile supplies, other than linen and gloves, are arranged on another sterilely draped back table. These tables are covered in part or in total with a sterile drape until the required items are in use or needed. Again, a separate table containing the supplies for the processing will be set-up for that procedure.

EQUIPMENT NEEDED FOR BONE MARROW RETRIEVAL

List of Equipment Needed

Retrieval Tray
Sterile Gloves
Sterile H2O
Ligature-Size 00
Sterile Gowns
#20 Knife Blades

Back Table Covers
Laparotomy Pack
4 x 8 Gauze
Linen Pack
Mortuary Pack

Styrofoam Shipper
Sterile Hand Towels
Autopsy Twine with Needle
Self Adhering Incision Drape

Contents of Trays

Harvest/Retrieval Tray
(Steam Sterilize)

4 x 8 Gauze Sponges
Self Retaining Balfour Retractor x2
Deaver Retractor x2
Storey Hemostats x6
Mallet - 2 lb. x2
#4 Knife Handle x2
Alexander Periostial Elevator x2
Lambotte Osteotome (Straight) - Small x2
- Medium x2
- Large x2
Lambotte Osteotome (Curved) - Small x2
- Medium x2
- Large x2
Lane Bone Holder x2

PREPARATION OF THE DONOR FOR STERILE TISSUE EXCISION

The handling and treatment of the donor by all personnel of the Tissue Bank will reflect the respect for the body after death. The procedures are to be carried out in the same ethical manner as all surgical procedures performed on the living. After determining that the donor meets all the donor criteria and permission is obtained from the next-of-kin and the medical examiner, if applicable, the donor is transported to the Tissue Bank.

Transporting the Donor to the Tissue Bank

The donor will always be transported from the morgue by two (2) technicians. Prior to transportation, careful identification of the donor must be made by the two technicians. Upon arrival in the Tissue Bank, the donor will be identified again by the Medical Officer and the Senior Technician. This is accomplished by comparing the name on the tags attached to the donor with the name on the SF 523B (authorization for Tissue Donation).

Preparation of the Anterior Surfaces

The anterior surfaces of the torso and the legs, from the clavicles to the mid thighs, are dry shaved using a razor. The body is then scrubbed with the Betadine solution and surgical scrub brushes for ten minutes and dried by blotting with dry sterile towels. The prepared surface is then sprayed with Betadine scrub.

Method for draping the donor for tissue excision

Draping of the donor will be accomplished by using a Laparotomy Pack and a Basic Pack. The draping technique to be used is the same as for the exploratory laparotomy procedure or a renal harvest procedure. A self adhering incision drape is recommended for use directly over the incision site.

HUMAN CADAVERIC BONE MARROW RETRIEVAL PROTOCOL

Method:

Unless the draping was left by the previous procedure (e.g., kidney harvest, etc.), draping shall be done with a basic laparotomy pack and one basic linen pack.

1. Using the self retaining Balfour retractor, retract the incision laterally, caudally, and cephalically (Figure 1).

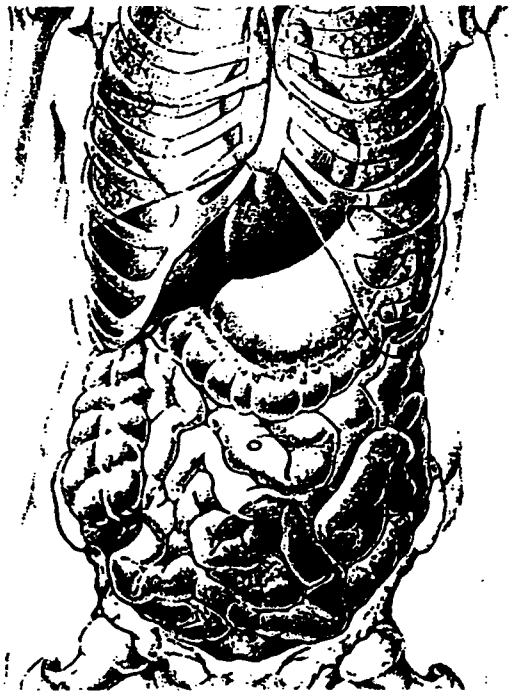
2. The Greater Omentum will now be exposed. Retract this cephalically and retain with the Balfour Retractor.

figure 1

3. Caudally, the Intestines will be exposed (Figure 2). Retract the intestines with a Deaver retractor. Cephalically, the Stomach, Liver, and Gall Bladder will be exposed. Retract these also with a Deaver retractor.

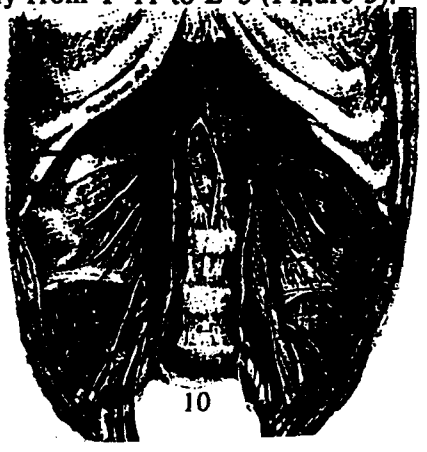
4. The lower thoracic and upper lumbar vertebrae will now be exposed. The Aorta and Vena Cava should have already been ligated during the previous harvest. If these vessels have not been ligated, clamp them off with the Storey hemostats and ligate with at least a size 0 silk suture.

figure 2



5. At this point, the vertebral column can be approached. Using a #20 knife blade on a #4 handle, incise the Anterior Longitudinal Ligament the length needed for the number of vertebral bodies you wish to harvest, usually from T-11 to L-5 (Figure 3).

figure 3



6. After this incision has been made, use an Alexander Periostial Elevator and strip this ligament to the lateral sides of the vertebrae.

7. Continue stripping this tissue until you can visualize the Transverse Process on the vertebrae.

8. With this exposure completed, use a straight Lambotte osteotome and disarticulate the vertebrae through the intervertebral disks at T-10 to T-11 and L-5 to S-1 (Figure 4).

9. With the vertebral cartilage disarticulated, take a large curved Lambotte osteotome and place it laterally on the pedicles that are located dorsally on the vertebrae.

10. With a mallet and the osteotome, create an osteotomy through the pedicles from T-11 to L-5 on the lateral sides of the vertebral column (Figure 5) using the Lane bone holder to steady the vertebral column during the osteotomies.

figure 4

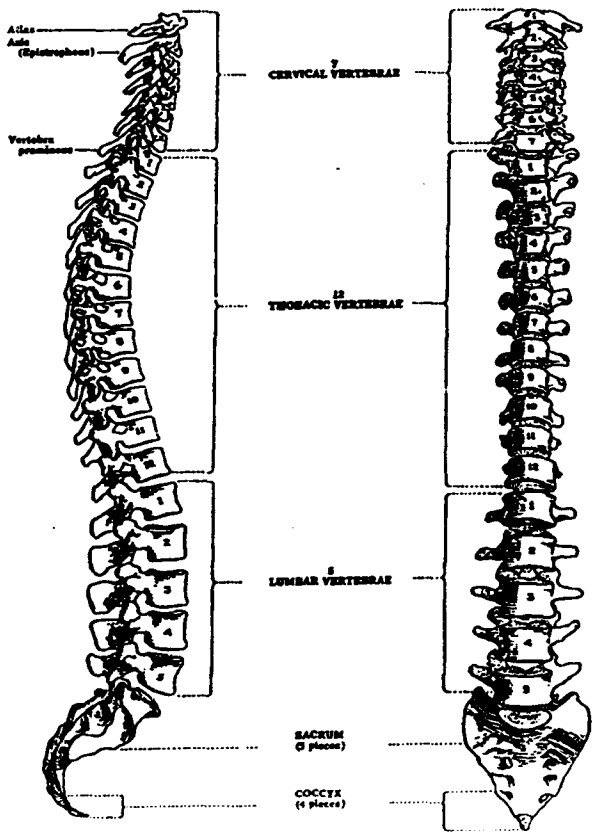
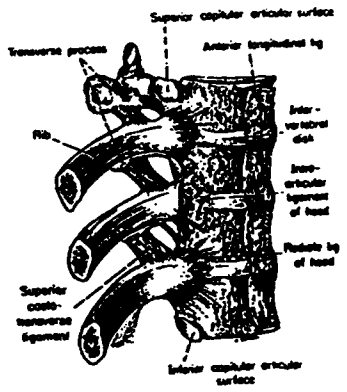


figure 5



11. With the osteotomies completed, you can now extract the vertebral log using the Lane bone holder.

12. Wrap the vertebral log in three sterile hand towels and place in a sterile, waterproof, plastic bag. Seal bag securely, thus ensuring an airtight package.

13. Place the package containing the vertebral log in a styrofoam shipping box (18x12x12 inches) that is capable of holding wet ice.

14. Cover the package with wet ice and seal the shipping box securely.

15. To close the surgical wound, remove all sponges and instruments from the wound and close the incisions with the autopsy twine and needles using a reverse running suture.

16. Remove all drapes and gear from the donor.

17. Wash the donor with either sterile H2O or 70% Isopropyl alcohol to remove the prep solution.

18. Wrap the donor in the standard mortuary pack with all appropriate identification tags attached.

19. Return the donor to the morgue.

SOLUTIONS REQUIRED FOR THE PROCESSING OF BONE MARROW

Preparation of Solutions

1. Iscoves:

   900 mls. containing Fungizone/Pen-Strep.
   100 mls. Fetal Calf Serum containing 100,000 units of DNase
   Osmolarity: 280-330 pH: 6.5-7.0

2. CPD-H:

   930 mls. Sterile 0.9% Sodium Chloride
   70 mls. Stock CPD-H Solution (filtered by 0.2 micron filter)
   Osmolarity: 280-320 pH: 6.5-7.0

3. CPD-H/EDTA:

   920 mls. Sterile 0.9% Sodium Chloride
   70 mls. Stock CPD-H Solution
   10 mls. Stock EDTA Solution
   EDTA pH: 6.8-7.0

Concentration of Solutions

1. Iscoves Solution with L-Glutamine (Hazelton Biologics): x10 liters

   a) Powdered Media - LOT #430-2200
   b) Fungizone (HEM Research Inc.) - 50 mls. 250 mcg/ml.
   c) Pen/Strep (Quality Biological Inc.) - 100 mls.
      Penicillin - 10,000 units/ml.
      Streptomycin - 10,000 mcg/ml.

2. CPD-H Solution: x500 mls.

   a) Tri-Sodium Citrate (Sigma) - 28.3 grams
   b) Dextrose (Sigma)- 34.7 grams
   c) Monobasic Sodium Phosphate (Sigma) - 2.22 grams
   d) Heparin (Sigma) (1000 units/ml.) - 71.4 mls.

3. EDTA Solution (Sigma): x100 mls.

   a) Disodium EDTA - 6 grams

4. DNase (Calbiochem): 1,000 units/ml.

EQUIPMENT NEEDED FOR BONE MARROW PROCESSING

List of equipment needed

Bone Marrow Tray	Sterile Gloves	Coupler Sites
Grinder Tray	Sterile Gowns	Transfer Tubing
#40 (35 mesh) Screen	#70 (65 mesh) Screen	Crimps
Back Table Covers	Sterile Tubing	50 cc Syringe
Receptacles - x3	600 ml. Bags	Large Basin
800 ml. Bags	1000 ml. Bags	Scale

Contents of Trays

Bone Marrow Tray
(Steam Sterilize)

1. 2 lb. Mallet
2. 3 lb. Mallet
3. Mayo Scissors
4. Kelly Hemostats
5. #3 Knife Handle
6. Straight Osteotome - x2
7. Curved Osteotome - x2
8. 4 x 8 Gauze Sponges
9. Sterile Hand Towels

Grinder Tray
(Steam Sterilize)

1. Grinder Plates - x2
2. Grinder and Parts

HUMAN CADAVERIC BONE MARROW PROCESSING PROTOCOL

Bone marrow will be submitted as a vertebral log wrapped in two (2) sterile hand towels, placed in a sterile plastic bag, wrapped again with a third sterile hand towel, and then placed in a second sterile plastic bag. The specimen is then placed into a styrofoam shipper and maintained on wet ice at four (4) degrees centigrade.

Three personnel are required for the proper disposition of the tissue; two technicians scrub for the procedure while a third circulates. All work will be performed in a sterile environment using aseptic technique.

Procedure for processing and freezing

1. Bone marrow package containing vertebral log is opened in operating room on a side table (not on field) being careful not to contaminate specimen.

2. Scrub tech #1 takes vertebral log and places it on the sterile field while scrub tech #2 assembles grinder.

3. Using an osteotome, remove all soft tissue and spinal processes from external surface. At this point, the vertebral log is disarticulated into individual bodies and maintained at room temperature (Figures 6 & 7).

figure 6

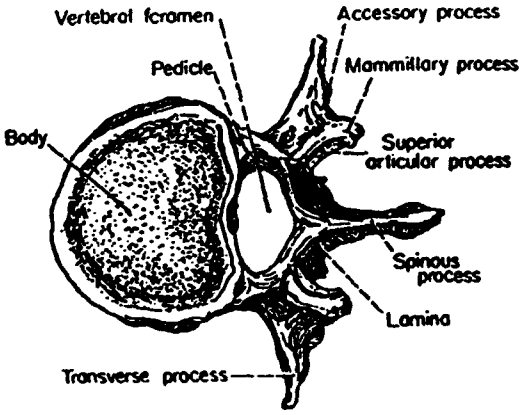
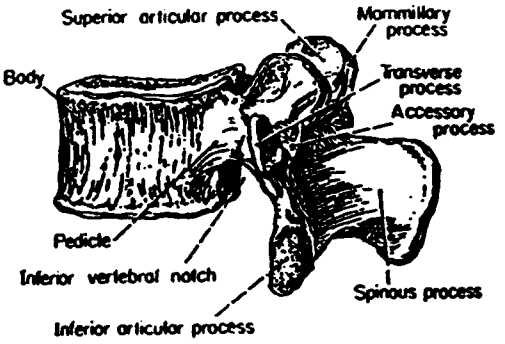


figure 7



4. Remove cartilaginous tissue and isolate the bone marrow matrix by removing the cortical plate (approximately 1/16 inch from the outer surface). BM (bone marrow) matrix from each vertebral body should be maintained in one liter of filtered CPD-H/EDTA saline solution at room temperature and processed as a group, not individually.

5. BM matrices are then sectioned into smaller segments and placed back into CPD-H/EDTA saline solution.

6. Isolate cells from the BM matrix by processing through the tissue grinder a minimum of two (2) times.

7. Bone marrow suspension is filtered through a No. 45 and a No. 70 screen stacked on top of a receptacle as marrow passes from grinder.

8. The suspension is then passed through a No. 45 and a No. 70 screen stacked on top of a receptacle to remove any unwanted sediment.

9. Pour suspension into a sterile basin containing the one liter of CPD-H saline solution. Volume of final cell suspension will be approximately two liters.

10. Using sterile tubing and a fifty (50) ml syringe, siphon twenty (20) mls. of suspension for aerobic and anaerobic cultures, counts, viabilities, and quality control culture maintained in a 37 degree centigrade incubator with 5% CO2.

11. Maintaining aseptic technique, siphon the remaining cell suspension from the basin into four 600 ml. bags in equal volumes (approximately 500 mls. each). Fold tubing over for spin, do not cut!

12. Four bags are then spun @ 2500 rpm for 10 minutes at 22 degrees centigrade.

13. Express supernatant off into two 1000 ml. transfer packs, crimp, cut, and then discard.

14. Gently resuspend cellular button with gauze in the 600 ml. bags then add coupler sites to each bag.

15. Using one liter of a combined Iscoves and Fetal Calf solution, resuspend two 600 ml. bags into one 800 ml. bag. Repeat this procedure with the remaining two bags. Crimp and cut the two 800 ml. bags, discarding the 600 ml. bags.

16. Place the two bags into a roller bottle and then in the walk-in incubator at 37 degrees centigrade for 1-2 hours until marrow is free of particulate matter.

17. After incubation, spike a 600 ml. bag into each 800 ml. bag and squeeze marrow into the 600 ml. bag for spin. Fold over the 800 ml. bag, do not cut!

18. Spin bags @ 2500 rpm for ten (10) minutes at 22 degrees centigrade.

19. Express supernatant back into the 800 ml. bags and crimp/cut. Discard supernatant.

20. Gently resuspend cellular button in the two 600 ml. bags with gauze.

21. Add coupler sites to bags and then add a bag of 75 mls. of plain Fetal Calf Serum to each bag and label as Bag A and Bag B.

22. Remove two mls. from each bag for aerobic and anaerobic cultures, quality control, counts, and viabilities.

23. Dispense cells to investigators according to counts received per ml of suspension.

24. After dispensing of cells, place marrow on ice to chill. After chilled (approximately 10 minutes), add 27% DMSO in a 50:85 ratio (DMSO to cell suspension) while gently agitating suspension on ice.

25. Using sterile technique, transfer marrow to 100 ml. or 50 ml. size freezing bags. Heat seal and cut tubing from bags and place in freezing tins.

26. Transfer remaining marrow to 2 ml. nunc tubes. Freeze either by Cryomed controlled rate freeze or place in a -80 degree centigrade freezer for one (1) hour. Upon completion of this cycle, transfer to a liquid nitrogen freezer for permanent storage.

CELL VIABILITY USING A HEMACYTOMETER

Materials Needed

1. Cell suspension
2. Hemacytometer and coverslip
3. Microscope
4. 20 lambda pipetter
5. Hand counter
6. Trypan Blue Solution (0.2%)

Method

The Trypan Blue exclusion technique is the most common method used to indicate the number or percentage of viable white blood cells in a given cellular suspension. Viable cells exclude the dye, while nonviable cells take up the dye, thereby fostering a visual distinction between unstained viable cells and blue-stained nonviable cells. After being stained with trypan blue, the cells must be counted within three (3) minutes; after that time viable cells begin to take up the dye.

The large squares are used for counting white blood cells. The middle area is used for counting red blood cells. The volume contained by each of these separate areas under a coverslip is 10E-4 ml. (in hemacytometers that are 0.1 mm deep). Therefore...

viable cells/ml. =

(average no. of viable cells in large sq.) x 10E4/ml. x 1/dilution;

% viable cells/ml. =

no. of viable cells/no. of viable cells + dead cells x 100%.

Procedure

1. For greater accuracy, agitate cell suspension prior to removing sample for viability check.

2. Take 20 lambda of trypan blue and 20 lambda of cell suspension and mix in a 96 well plate. Aspirate in an up and down motion to ensure optimal dilution.

3. Take 20 lambda of this and load into a hemacytometer and count the number of viable white blood cells.

USING THE COULTER COUNTER

Start-up

When turning the coulter on, always remember to run a background check first. This is simply a clean vial with isotonic saline. A reading of 0-50 is an acceptable range prior to running samples through the counter.

Method

1. Hold a sample tube containing suspension to be counted under the aspirator tip so the tip is completely immersed in the sample. This eliminates the possibility of air entering the aspirator tip along with the sample. Do not, however, allow the tip to come into contact with the bottom of the sample container.

2. Press the control bar keeping the tip immersed until the DISPENSE lamp illuminates (approximately 4 seconds).

3. Remove the sample container and wipe the exterior of the tip with a Kimwipe.

4. Using a clean vial, press the control bar momentarily to dispense the dilution into the vial. During delivery, approximately 2.5 mls. of dilutent is applied to the aspirator tip to wash out the inside surface. To prevent carry-over, draw the vial downward as the liquid level in the vial rises toward the aspirator tip. Do not submerge the tip in the dispensed sample. Do not remove the vial from the dispenser until the DISPENSE lamp turns off (about 7 seconds).

5. After the DISPENSE lamp turns off, remove the vial and wipe the aspirator tip as described in step 3.

6. If the sample contains RBC's, use six (6) drops of lysing solution, Zap-oglobin II, cap sample vial, agitate, and let sit for 2 minutes.

7. Place the vial containing the diluted and lysed sample on the Sampling Stand platform. Aperture tube with adjoining reflective mirror should be situated near the bottom of the vial but not resting on its bottom.

8. Turn the counting knob located on the sampling stand clockwise to "RESET" and wait for light to come on within the stand and a scatter pattern to appear within the aperture monitor. Turn knob to "COUNT".

The following Coulter Counter settings should be used at all times:

CURRENT - 220
FULL SCALE - 1
POLARITY - AUTO
LOWER THRESHOLD (Tl) - 10.0
UPPER THRESHOLD (Tu) - 99.9
ALARM THRESHOLD (ms) - OFF
ATTENUATION - 4
PRESET GAIN - 8
MANOMETER SELECT - 500 ul
STIRRER CONTROL - OFF

If there are any questions or problems that arise while attempting to use the Coulter, please refer to the Operations Manual.

FREEZING CELLS IN THE CRYOMED CONTROLLED RATE FREEZER

The following steps must be followed

1. Turn main power switch to "ON" position; wait 10 minutes for warm-up, then open valve on LN2 tank and secure door.

2. Check "START TEMP" dial (0 degrees centigrade).

3. Push "CONTROL READY" button - hold in for 2 seconds.

4. Turn on "COOLANT" and "BLOWER" switches.

5. Turn on "POWER" and "CHART" switches located on recorder panel.

6. Turn TC monitor to "CYCLE" position.

7. Check the following dials and settings:

   a. LIQUID PHASE SECTION

      1) When all is checked, the "PUSH TO START" button is activated.
      2) "FREEZING RATE" - set at 1.
      3) "START PHASE CHANGE" - set at -3 degrees.

   b. PHASE CHANGE SECTION

      1) "TEMP DROP" - set at -65 degrees.
      2) "END PHASE CHANGE" - set at -34 degrees.

   c. SOLID PHASE SECTION

      1) "FREEZING RATE" - set at 5 degrees.
      2) "END TEMP" - set at -38 degrees.

   d. SOLID PHASE II SECTION

      1) "FREEZING RATE" - set at 10 degrees.
      2) "END TEMP" - set at -80 degrees.

   e. ALARM SECTION

      1) Switch to "ON" position.

When "RECORDER" indicates that chamber is at "START TEMP" (0 degrees), it is time to load cells into freezer.

1. Switch "BLOWER" and "COOLANT" switches to the "OFF" position.

2. Release freezer unit door latch.

3. Insert the TC (thermocoupler) probe into the control sample vial - a clamp or hemostat may be needed to remove plastic cork from neck of old vial.

4. Replace control sample vial in center of rack of cells.

5. Close door of freezer unit and latch shut.

6. Switch "BLOWER" and "COOLANT" to "ON".

7. When recorder indicates internal temperature has returned back to start temperature (0 degrees), and the "CONTROL READY" light is on, press the "PUSH TO START" button. Freeze is automatic from this point on.

8. When alarm sounds and "END TEMP" light comes on, freeze is done. Turn alarm, blower, and coolant switches off. Then unlatch door, remove control vial, and turn LN2 tank valve to closed position. Turn machine switches (power, recorder, and alarm) off.

9. Rinse thermocoupler probe in diluted hypochlorite solution to remove any contaminants.

THAWING BONE MARROW NUNC TUBES

Materials

1. Two 50 ml. conical tubes; one empty and one with 30 mls. of 20% Fetal Calf Serum in Iscoves (thawing media) - all sterile.

2. DNase diluted to 5,000 units/ml.; 0.3 ml. of this will be needed for each tube.

3. Sterile pipettes, 2 ml. and 10 ml., and a pipetting device.

4. Bucket of dry ice with a tight cover.

5. Frozen bone marrow tubes (keep in the dry ice).

Method

1. Remove outer ice from tube using either a water bath or hot running tap water.

2. Thaw tube by slowly agitating through the water.

3. When thawed, spray nunc tube with 70% Isopropyl alcohol and wipe.

4. Remove cells sterilely from the tube with a pipette and place into an empty 50 ml. conical tube.

5. Slowly drip 30 mls. of thawing media (20% Fetal Calf Serum in Iscoves) into the cells.

6. This is the end of the actual thaw; the slow dripping is designed to change the osmolarity of the cells (their liquid environment) gradually, so as not to traumatize them too much in their delicate condition (total time should take at least ten minutes).

7. Incubate the tube of cells (capped, upright) in a 37 degree incubator for thirty (30) minutes.

8. Remove from the incubator and place under a laminar flow hood. Add 1500 units of DNase (50 units/ml.) to the cells and reincubate the tube for 60 minutes.

9. Centrifuge the tube(s) @ 1000 rpm for 10 minutes at room temperature, decant supernatant into a third sterile 50 ml. conical tube, and set it aside. Gently agitate the tube(s) of cells, shaking the cell button to try to get it to go into solution, or see if it is clotted.

10. **IF CLOTTED**: Pour supernatant with DNase back into cells, cap tube and reincubate overnight. Slowly pipette cells up and down to break up this cell pellet prior to doing so.

11. **IF NOT CLOTTED:** Resuspend cells in bone marrow culture media, take a small sample to verify count and viability, and place in appropriate sized flask at a final concentration of 1-5 X 10E6 cells/ml. and incubate at 37 degrees centigrade.

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CLINICAL RECORD	AUTHORIZATION FOR AUTOPSY

In the event authorization for autopsy is obtained by letter, telegram, voice recorded or monitored telephone call, paragraphs 1, 2, and 3 shall be completed by medical facility authorities and the letter, telegram, voice recording or memorandum confirming telephone call of authorization attached to this form for permanent file.

1. NAME AND LOCATION OF MEDICAL FACILITY	DATE AND TIME

2. I (We) request and authorize the physicians in attendance at the above named medical facility to perform a complete autopsy on the remains of _____ _____. I (We) understand that a complete autopsy may include, but not be limited to, examination of the head, eyes, spinal cord, chest, abdomen and extremities unless excluded under restrictions hereinunder, and I (We) authorize the removal and retention or use for diagnostic, scientific, or therapeutic purposes any parts, tissues, or organs as such physicians or their designees may deem proper, and the final disposal thereof in such manner as may be prescribed by competent authority (Commanding Officer, Medical Director, etc.) in this facility.

This authority is granted subject to the following restrictions: _____

_____

_____

_____

(If No Restrictions, Write "None")

The following special examinations are requested: _____

_____

_____

3. I (We) represent that I am (we are) the _____
(Relationship/Authority)

of the deceased and entitled by law to control the disposition of the remains.

Signed _____

WITNESSES (medical facility staff members):

Signed _____

Signed _____
(Name and Title)

Signed _____
(Name and Title)

FOR ADMINISTRATIVE USE ONLY

Case falls within jurisdiction of Medical Examiner/Coroner . . . . . YES ☐ NO ☐

Medical Examiner/Coroner released remains from his jurisdiction to this authority . . . . . YES ☐ NO ☐

SIGNATURE	TITLE	DATE

PATIENTS IDENTIFICATION *(For typed or written entries give: Name—last, first, middle; grade; date; medical facility)*	REGISTER NO.	WARD NO.

AUTHORIZATION FOR AUTOPSY
Standard Form 523 (Rev. 10-75)
Prescribed by General Services Administration and Interagency Comm. on Medical Records
FPMR 101-11.806-8 — 523-108

Standard Form 523-B

CLINICAL RECORD | AUTHORIZATION FOR TISSUE DONATION

In the event authorization for tissue donation is obtained by letter, telegram, or mechanically recorded telephone call, paragraphs 1 and 2 shall be completed by hospital authorities and the letter, telegram, or memorandum confirming telephone call of authorization attached to this form for permanent file.

NAME AND LOCATION OF HOSPITAL	DATE
1.	

2. You are hereby authorized to remove the following-named tissue from the remains of

..... for donation to the Tissue
(Name of deceased)

Bank of .....
(Name of hospital)

.....
(Specify tissue: e. g., bone, artery, cartilage, skin, fascia, dura, nerve, tendon, etc.)

Authority is also granted to use the tissue in grafts upon living persons, or to dispose of the tissue in a suitable manner.

Signature of witness .....

Address .....

.....

Signature .....
(Person authorized to consent)

Address .....

.....

Authority to consent .....

PATIENT'S IDENTIFICATION (*For typed or written entries give Name—last, first, middle; grade; date; hospital or medical facility*)

REGISTER NO.	WARD NO.

AUTHORIZATION FOR TISSUE DONATION
Standard Form 523B
523-305

General Services Administration and
Interagency Committee on Medical Records
FPMR 101-11.809-3
October 1974

Appendix B